SUPPLEMENTARY APPENDIX

Optimal interleukin-7 receptor-mediated signaling, cell cycle progression and viability of T-cell acute lymphoblastic leukemia cells rely on casein kinase 2 activity

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Supplemental Data

Supplemental Methods

Figures S1-12

Supplemental Methods

Cell culture and experimental conditions. Primary leukemia and TAIL7 cells were cultured as 2×10^6 cells/ml in RPMI 1640 culture medium supplemented with 2mM L-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin (Life Technologies), and 5% (vol/vol) heat-inactivated FBS (PAA Laboratories GmbH). TAIL7 cells were maintained in culture medium plus 10ng/ml rmIL-7 (PeproTech EC). DND-41 and HPB-ALL cells were cultured as 0.5 and 1×10^6 cells/ml respectively, in similar culture medium but supplemented with 10% heat-inactivated FBS without IL-7. Cells were maintained at 37°C in 5% CO2. TAIL7 and HPB-ALL cells were IL-7-deprived in medium without FBS or with 1%, respectively for 24h prior to incubation with the CK2 pharmacological inhibitor CX-4945 (kindly provided by Cylene Pharmaceuticals) or TBB (Merck-Millipore). T-ALL cells were cultured in medium RPMI-1% FBS with or without IL-7 and inhibitor, at the indicated concentrations and time points. HEK293T cells were maintained in DMEM (Gibco) supplemented with 10% FBS, L-glutamine and penicillin streptomycin.

RNA extraction, reverse transcription-PCR and semiquantitative RT-PCR RNA was extracted using a Trizol purification method. For the reverse transcription-PCR, up to 1 mg of total RNA was reverse transcribed using SuperScript II (Invitrogen) and random hexamers. Expression of each gene was normalized to the expression levels of 18S expression. Primers used for the qRT-PCR are the following: for forward step CK2alpha (5'-AAGACCCTGTGTCACGAACC-3'), CK2alpha' (5'-AAAAGCTGCGACTGATAGATTGG-3'), CK2beta (5'-CCAGGCTCTACGGTTTCAAG-3'), 18S (5'-CGCGGCTGCTGGCACCAGACTT-3')

and for reverse step CK2alpha (5'-GCCAAACCCCAGTCTATTAGTC-3'), CK2alpha' (5'-GAGGCTACACGAACATTGTACTC-3'), CK2beta (5'-CCCACCACAATAACGACTCC-3') and 18S (5'-CGCGGCTGCTGGCACCAGACTT-3'). All the amplifications were performed in a Viia7 System thermocycler (Applied Biosystems).

Immunoprecipitation TAIL7 cells were washed in PBS then lysed with RIPA Buffer. For each condition 125μg of total protein were used to procced with the protocol. Specific antibodies for IL-7R, CK2alpha (Santa Cruz Biotechnology) and IGG (Peprotech), in equal amounts, were added to each experimental sample and incubated overnight in cold with rotation. Protein A/G beads (Santa Cruz Biotechnology) were added and incubated for 3h30m in cold with rotation. The beads were washed and equilibrated in RIPA buffer for 1h in cold with rotation, prior to use. Remove the supernatant and wash the beads 3 times with RIPA buffer. Ressuspend the beads in 50μl of RIPA buffer and run the samples in a SDS-PAGE gel.

Synergism determination. T-ALL cells were cultured as previously described and incubated with increasing concentrations of CX-4945 alone or in combination with pan-JAK inhibitor (JAK inhibitor I/ Pyridone 6/ CMP6, Calbiochem) or Ruxolitinib (Axon Medchem). The concentration of the inhibitors in combinations were determined by a constant ratio of 3:1 (CX-4945:JAK inhibitor). All calculations of synergism were performed by CalcuSyn Software (Biosoft). The combination index (CI) and the median-effect dose (Dm) calculations are based on Chou and Talalay equations for quantification of synergism or antagonism of two drugs. The values of CI should be

considered as following: <1 indicates synergism, =1 indicative of additive effect, and >1 represents antagonism of the two drugs.

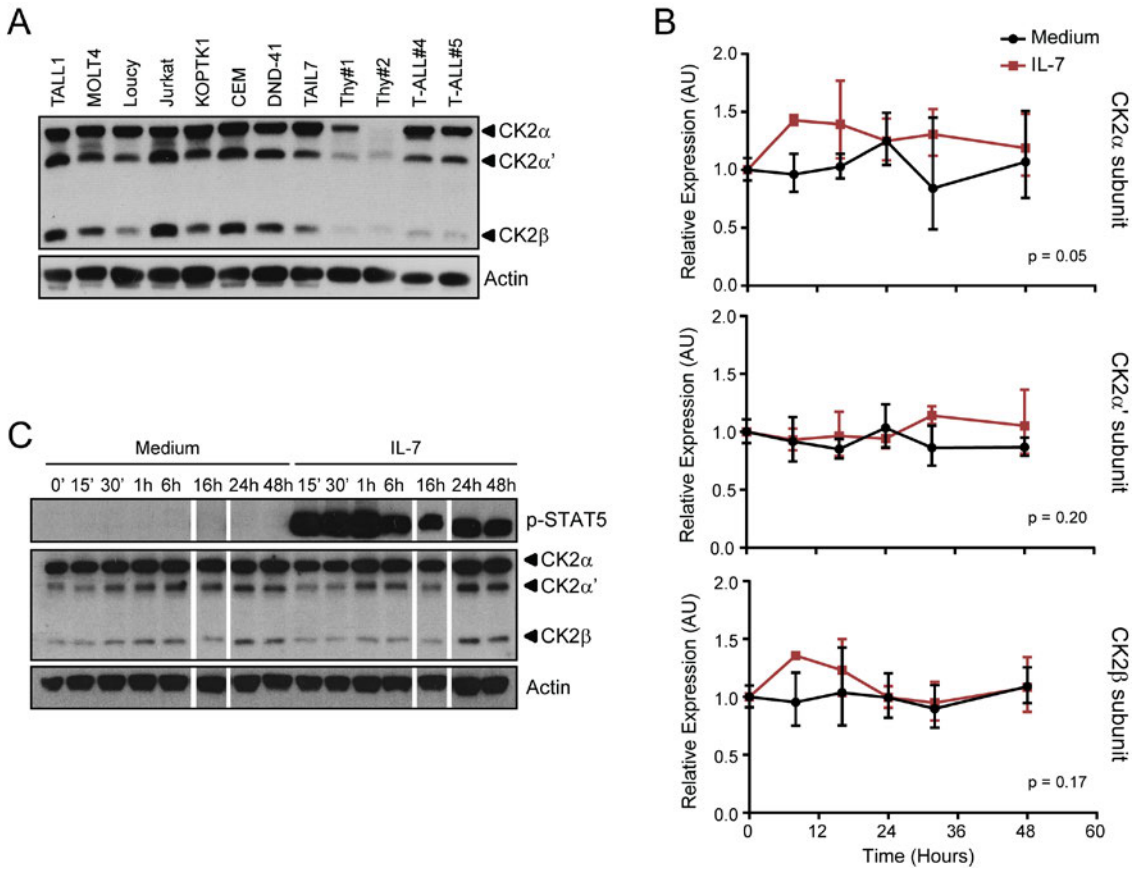

Figure S1. CK2 expression determination. CK2 basal protein expression **(A)** was measured in several T-ALL cell lines, primary T-ALL samples (T-ALL #4 and #5) and normal thymocytes (Thy #1 and #2) by immunobloting. TAIL7 cells were incubated with IL-7 (20ng/ml) for the indicated time points and mRNA **(B)** and protein **(C)** levels of CK2 subunits were determined by qRT-PCR and immunoblotting, respectively. Paired t-test was performed.

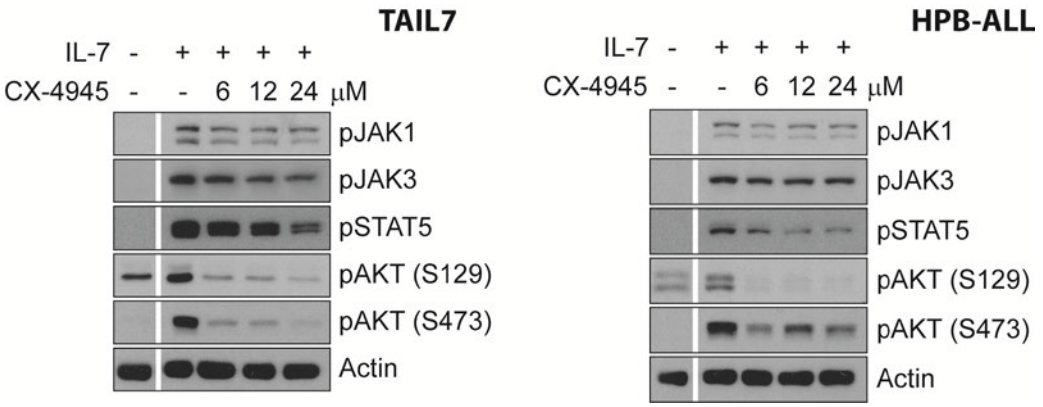

Figure S2. CK2 inhibition using CX-4945 prevents IL-7-mediated viability at low levels TAIL7 and HPB-ALL cells pre-incubated for 2 hours with CX-4945, at increasing concentrations as indicated, and stimulated with IL-7 (50ng/ml) for 15 minutes. Total protein extracts were resolved by SDS-PAGE, phosphorylated proteins were detected as described in 'Methods'

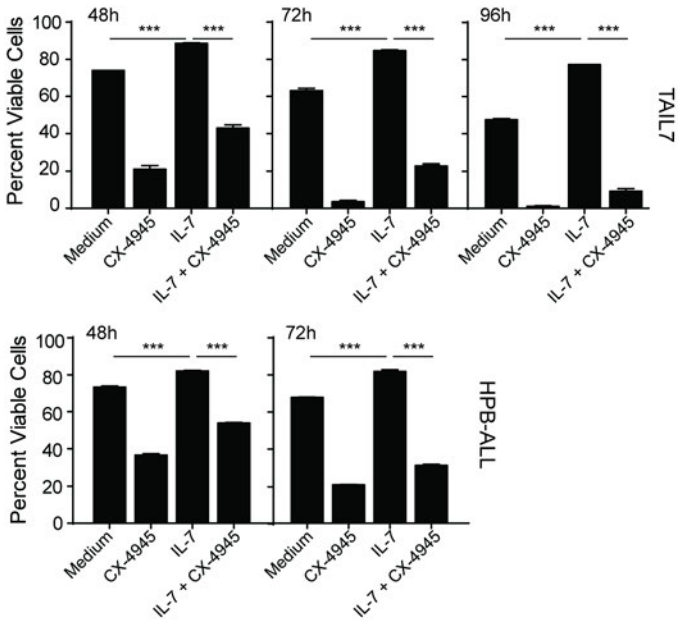

Figure S3. CK2 inhibition prevents IL-7-mediated viability of T-ALL cells. TAIL7 and HPB-ALL cells were incubated with CX-4945 (6μM) and stimulated with IL-7 (20ng/ml) for the indicated time points. Viability was measured by flow cytometry FSCxSSC discrimination as described in 'Methods'. Representative experimental data is shown as mean±sem of 3 replicates. *** P<0.001 comparing with the IL-7 condition (Two-way ANOVA, with Bonferroni's post-test)

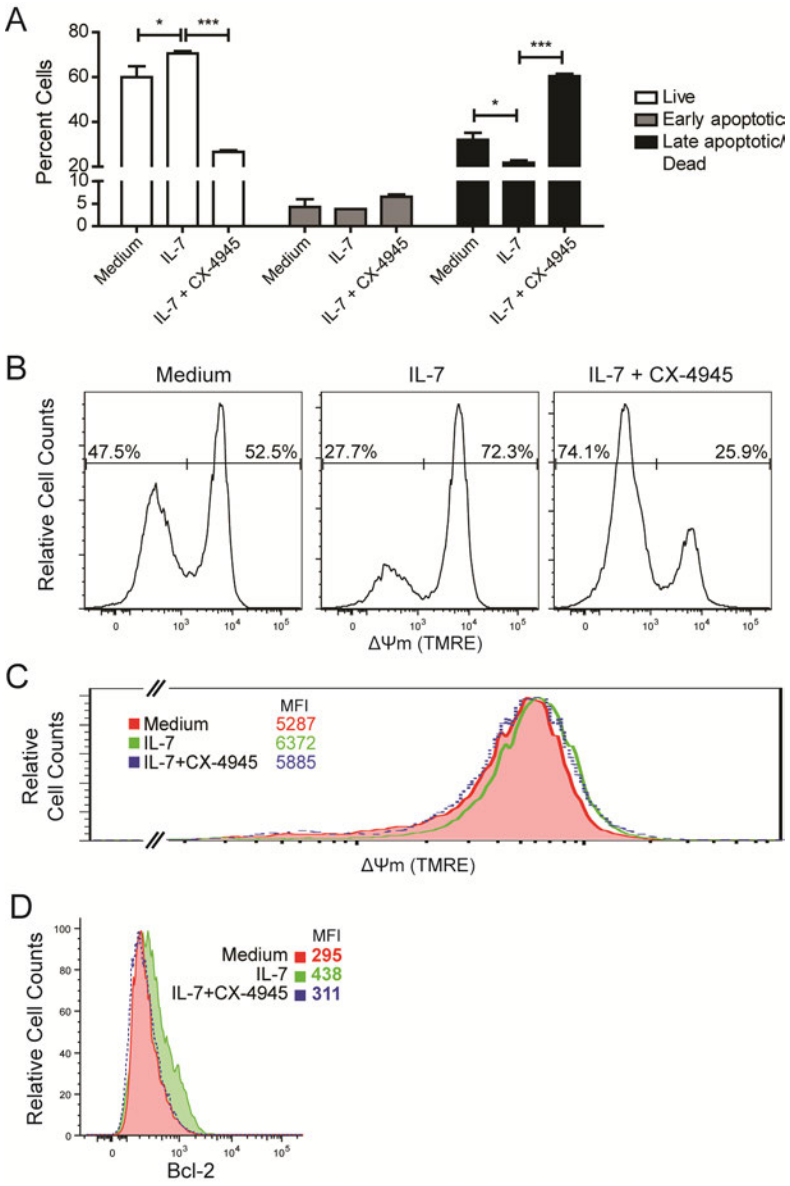

Figure S4. CK2 inhibition using CX-4945 prevents IL-7-mediated viability of HPB-ALL cells. (A) Apoptosis of HPB-ALL cells was measured by Annexin V and 7AAD staining after 72h of culture with IL-7 (20ng/ml) in the presence or absence of CX-4945 (6μM). A representative experiment out of 3 performed is shown. Mitochondrial membrane potential (ΔΨm; TMRE) was determined at 48h within the whole cell population **(B)** and live cell population **(C)** as described in 'Methods'. Bcl-2 levels were analyzed by flow cytometry at 48h **(D)**.

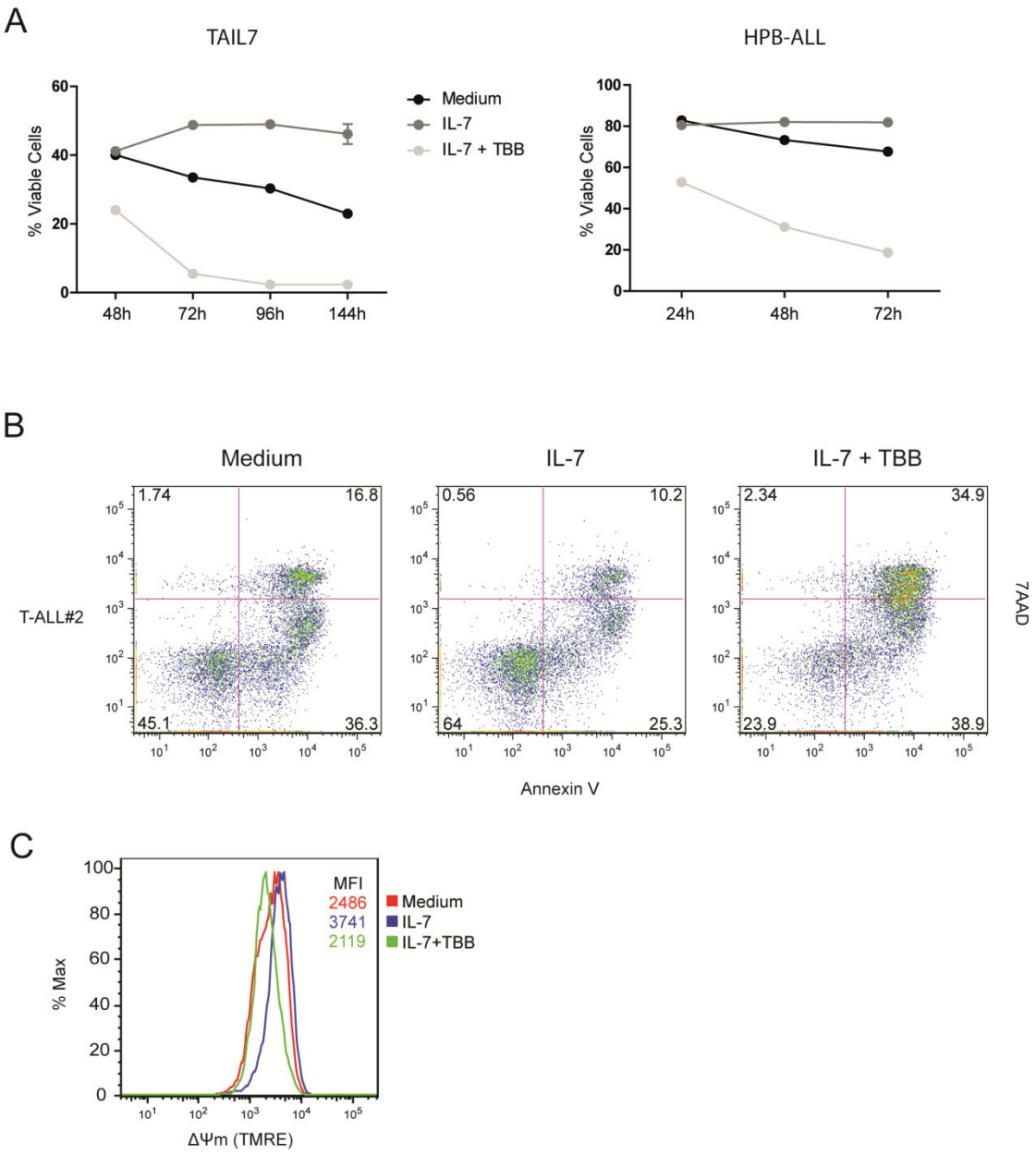

Figure S5. CK2 inhibition using TBB prevents IL-7-mediated viability of T-ALL cells. (A) Viability of TAIL7 and HPB-ALL cells was measured by FSC x SSC flow cytometry analysis at the indicated time points in the presence and/or absence of IL-7 (20ng/ml) and TBB (30µM). Data is shown as mean±sem of 3 replicates. **(B)** T-ALL primary cells were stimulated with IL-7 and/or treated with TBB. Apoptosis was assessed after 48h or 72h of culture by Annexin V/7AAD staining. A representative case out of 3 analyzed is shown. **(C)** Mitochondrial membrane potential (ΔΨm; TMRE) in TAIL7 cells was determined within the live cell population as described in 'Methods'.

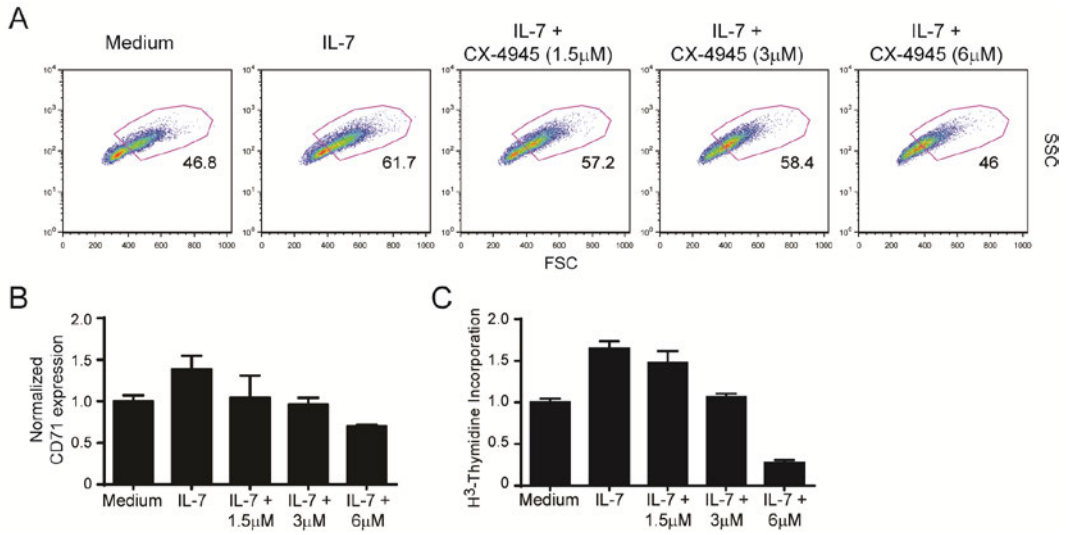

Figure S6. CK2 inhibition impairs IL-7-mediated T-ALL cell growth and proliferation in a dose-dependent manner. TAIL7 cells were cultured for 72h under the indicated conditions, with increasing concentrations of CX-4945. **(A)** Cell size increase was determined by analysis of FSC distribution by flow cytometry. **(B)** Expression of CD71 at the surface of TAIL7 cells was measured by flow cytometry. **(C)** Proliferation of TAIL7 cells was assessed by ^{3}H-thymidine incorporation as described in 'Methods'. Representative data from 2 independent experiments is shown as mean±sem of 2 replicates.

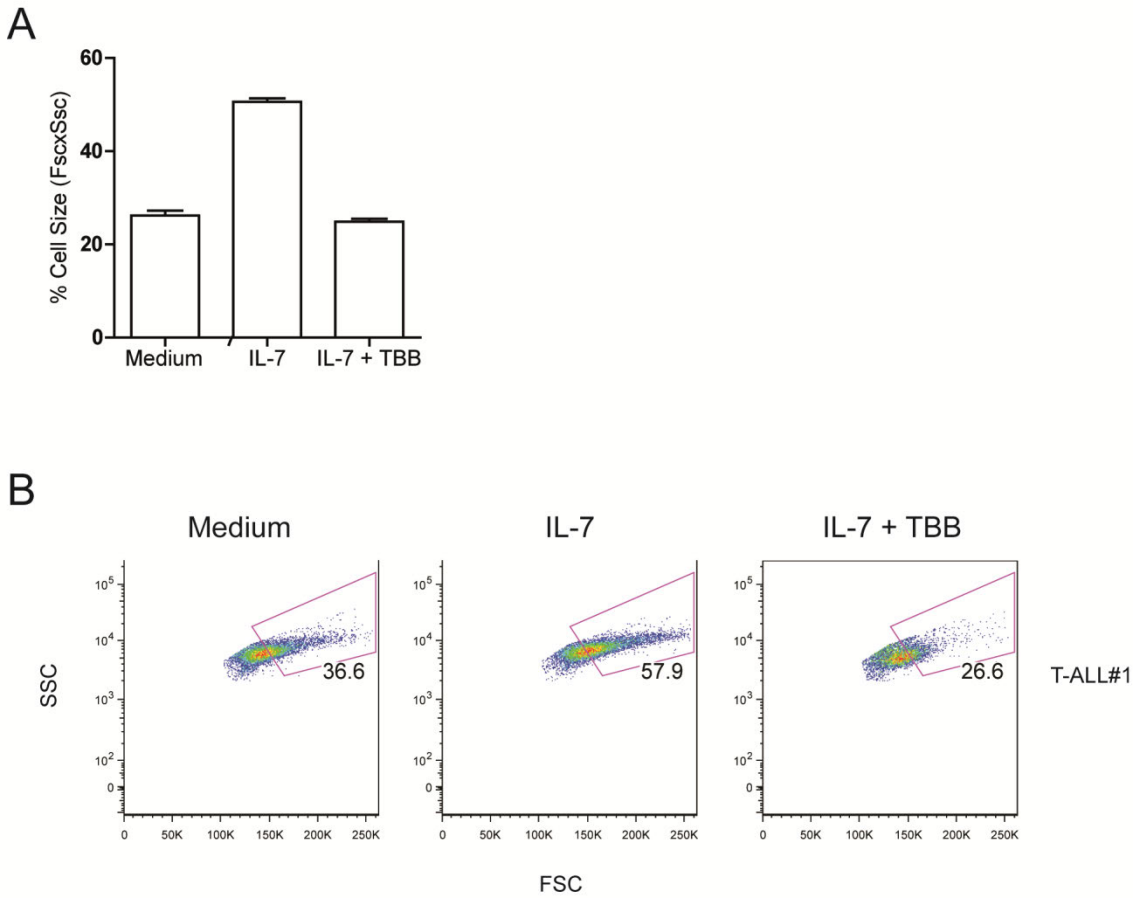

Figure S7. CK2 inhibition using TBB abrogates IL-7-mediated T-ALL cell growth. TAIL7 **(A)** or primary T-ALL **(B)** cell size increase (cell growth) was determined by analysis of FSC distribution by flow cytometry after 48h or 72h with IL-7 (20ng/ml) and/or CK2 inhibition with TBB (30µM).

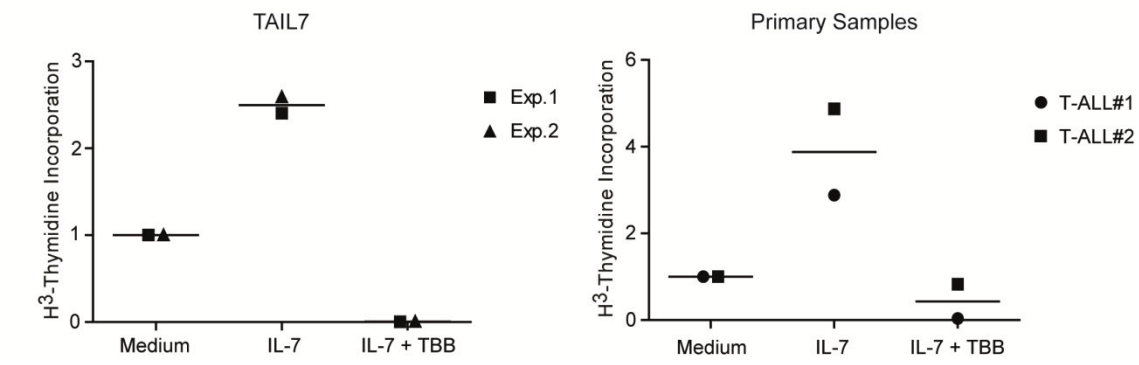

Figure S8. CK2 inhibition using TBB abrogates IL-7-mediated T-ALL cell proliferation. Proliferation of TAIL7 **(A)** or primary T-ALL **(B)** cells was determined by ^{3}H-thymidine incorporation as described in 'Methods', here represented as normalized values to the Medium control condition.

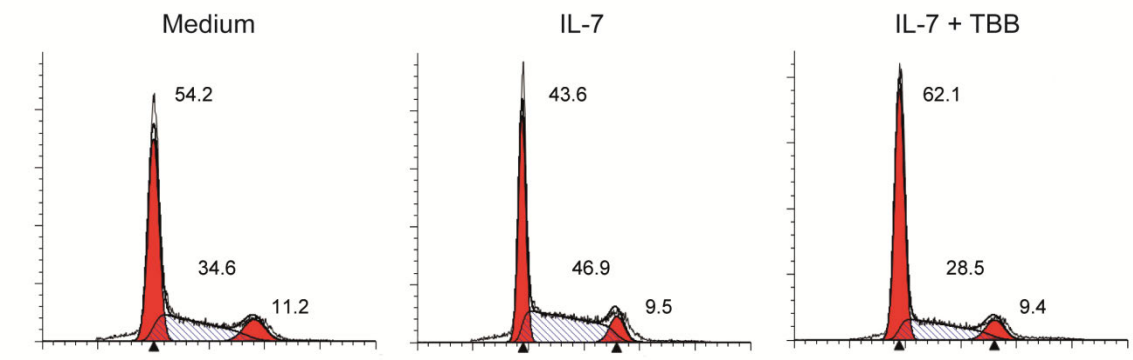

Figure S9. CK2 inhibition using TBB prevents IL-7-mediated T-ALL cell cycle progression. TAIL7 cell cycle profile was determined after 48h incubation with IL-7 stimulation (20ng/ml) and TBB treatment (30μM), as described in 'Methods'.

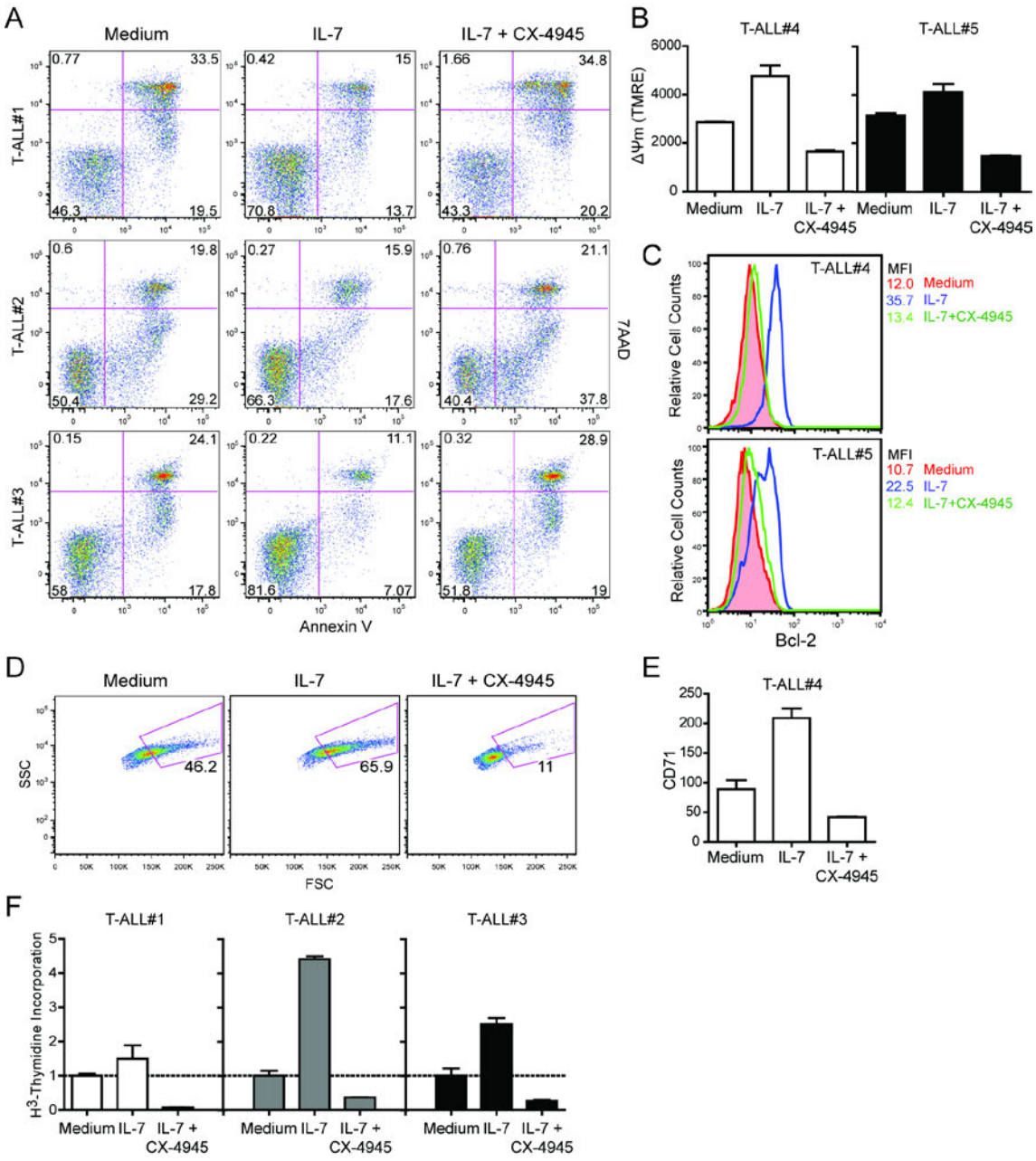

Figure S10. CK2 inhibition abolishes IL-7-mediated viability, activation and proliferation of primary T-ALL blasts from diagnostic pediatric patients. Primary T-ALL cells were cultured in medium alone or with IL-7 (20ng/ml) in the presence or absence of CX-4945 (6μM) and analyzed at 48h or 72h for IL-7 mediated effects. Viability and apoptosis were assessed by Annexin V/7AAD staining **(A)**, TMRE **(B)** and Bcl-2 expression **(C)**. 'Activation' status was assessed by cell size determination **(D)** and CD71 surface expression **(E)**. **(F)** Proliferation was determined by ^{3}H-thymidine incorporation.

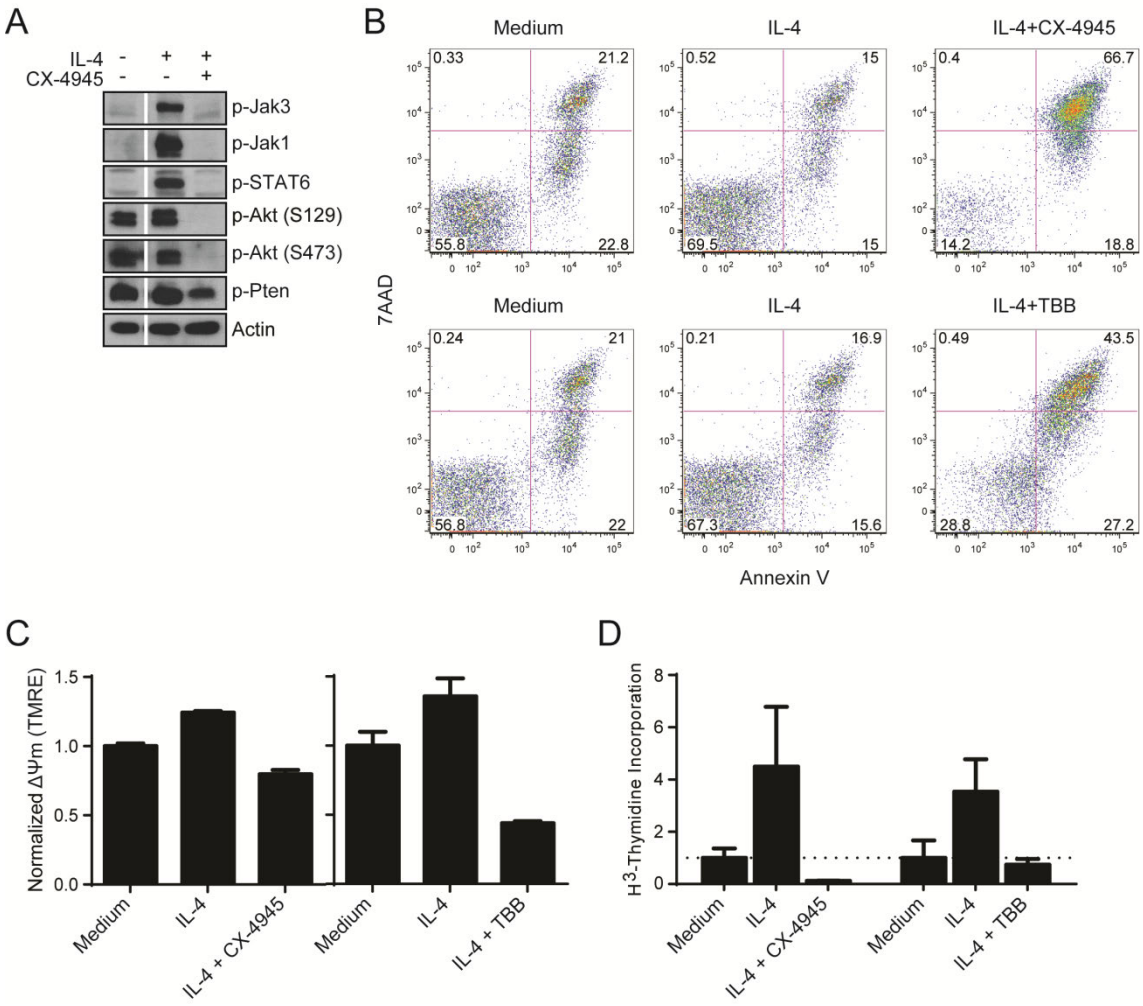

Figure S11. CK2 inhibition prevents IL-4-mediated viability of T-ALL cells. (A) TAIL7 were pre-incubated for 2 hours with CX-4945 (12µM) and stimulated with IL-4 (50ng/ml) for 15minutes. Total protein extracts were resolved by SDS-PAGE, phosphorylated proteins were detected as described in 'Methods. **(B)** Viability and apoptosis of TAIL7 cell was measured by Annexin V/7AAD staining at 72h in the presence and/or absence of IL-4 (20ng/ml), CX-4945 (6µM) or TBB (30µM). Representative experimental data is shown as mean±sem of 3 replicates. **(C)** Mitochondrial membrane potential (ΔΨm; TMRE) in TAIL7 cells was determined within the live cell population as described in 'Methods'. **(D)** Proliferation of TAIL7 cells was determined by ^{3}H-thymidine incorporation as described in 'Methods' here represented as normalized values to the Medium control condition.

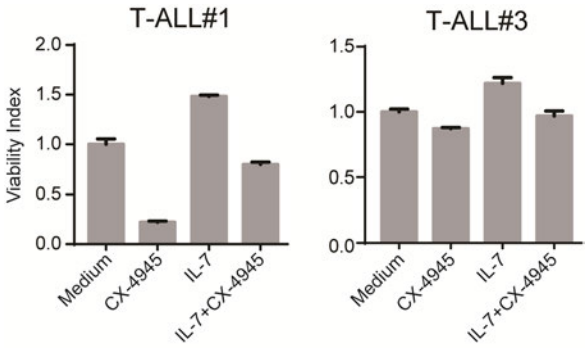

Figure S12. CK2 inhibition prevents IL-7-mediated viability of T-ALL primary cells. Primary samples of T-ALL patients were incubated with CX-4945 (6μM) and stimulated with IL-7 (20ng/ml) for 72h. Viability was measured by FSCxSSC discrimination as described in 'Methods'. Viability index was determined as medium condition as control. Results are summarized as mean±sem of experimental triplicates.